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<b>(21) International Application Number:</b> PCT/US96/11096 <b>(22) International Filing Date:</b> 28 June 1996 (28.06.96)  <b>(30) Priority Data:</b> 60/000,721 30 June 1995 (30.06.95) US  <b>(71) Applicant:</b> DNA PLANT TECHNOLOGY CORPORATION [US/US]; 6701 San Pablo Avenue, Oakland, CA 94608 (US).  <b>(72) Inventors:</b> BEDBROOK, John, R.; 160 La Salle Avenue, Piedmont, CA 94610 (US). DUNSMUIR, Pamela; 160 La Salle Avenue, Piedmont, CA 94610 (US). HOWIE, William, J.; 2847 Avon Avenue, Concord, CA 94520 (US). JOE, Lawrence, K.; 8101 Phaeton Drive, Oakland, CA 94605 (US). LEE, Kathleen, Y.; 3992 Lyman Road, Oakland, CA 94602 (US).  <b>(74) Agents:</b> BASTIAN, Kevin, L. et al.; Townsend and Townsend and Crew L.L.P., Two Embarcadero Center, San Francisco, CA 94111-3834 (US).		<b>(81) Designated States:</b> AU, CA, JP, MX, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> DELAYED RIPENING TOMATO PLANTS  <b>(57) Abstract</b>  The present invention provides tomato plants exhibiting a delayed ripening phenotype. The plants of the invention comprise a T-DNA insert comprising a truncated <i>Acc2</i> gene. Integration of the T-DNA insert into the plant genome inhibits ethylene biosynthesis in the fruit.		

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## Delayed Ripening Tomato Plants

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### BACKGROUND OF THE INVENTION

The present invention relates generally to the breeding of tomato plants. More specifically, the invention relates to the introduction of a transgene that confers a delayed ripening phenotype on tomato plants.

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The plant hormone ethylene has a profound influence on plant physiology. Active in trace amounts, it affects a number of processes such as fruit ripening, seed germination, plant growth, leaf and flower senescence, pathogen infection, and the interaction of plants with their environment. In particular, ethylene induces a number of physiological changes associated with fruit ripening such as accumulation of carotenoid pigments, conversion of chloroplasts to chromoplasts, the increased expression of genes encoding cell wall degradation enzymes, fruit softening and susceptibility to pathogens.

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Control of the effects of ethylene is a particularly useful approach to controlling fruit ripening in tomato. More than 80% of tomatoes (by volume) currently sold in the United States are picked while green. Growers harvest green tomatoes for several reasons: (1) green tomatoes are firmer, enabling them to withstand shipping and handling with less injury; (2) a green tomato harvest is less labor-intensive and less costly than a vine-ripe harvest and (3) green fruit stays in the field for a shorter period of time reducing the risk of loss from weather or pests.

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After harvest, either the packer or the repacker exposes the green tomatoes to an external source of ethylene gas to cause the tomatoes to develop red color. This practice reddens the fruit, but these tomatoes will not always develop full flavor when picked at the green stage. Fruit that have begun to ripen need to be shipped at a low temperature to delay ripening, however these low temperatures decrease fruit quality. Frequently, a portion of the green fruit is harvested at the immature green stage, which means that it will never achieve full ripeness even with the application of ethylene. Such tomatoes, together with other distribution-damaged tomatoes, do not achieve full flavor potential and, we believe, are a major factor contributing to consumer dissatisfaction with current fresh market tomatoes.

In tomato (and other so called climacteric fruit), fruit ripening is associated with a burst of respiration and a concomitant increase in ethylene production. Once ripening is initiated, the endogenous ethylene production rises autocatalytically.

If ethylene production could be controlled, tomato fruit could be left on the vine longer to develop the fruit components which contribute to flavor. The present invention addresses these and other needs.

### SUMMARY OF THE INVENTION

The present invention provides tomato plants comprising a genetic locus having a sequence substantially identical to SEQ. ID. No. 1. The plants bear fruit that display significant ripening impairment once the breaker stage is reached. In particular, the fruit reach pink stage about 2 to about 3 weeks after breaker stage, when the fruit are picked at breaker stage and stored at 15°C. Preferred plants are germinated from seed deposited with the American Type Culture Collection under Accession No. \_\_\_\_\_.

The invention further provides tomato fruit from the plants of the invention. The fruit of the invention have ethylene levels less than about 1.0 nl/g/hr, preferably less than about 0.5 nl/g/hr.

The invention also provides methods of tomato plants with decreased ethylene production. The methods comprise crossing a parent tomato plant with a tomato plant comprising a genetic locus of the invention. The tomato plant comprising the genetic locus can be a plant germinated from seed deposited with American Type Culture Collection under Accession No. \_\_\_\_\_. The method may further comprise the step of selecting progeny bearing fruit that reach pink stage about 2 to about 3 weeks after breaker stage, when the fruit are picked at breaker stage and stored at 15°C.

### Definitions

The phrase "nucleic acid sequence" refers to a single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. It includes both self-replicating plasmids, infectious polymers of DNA or RNA and non-functional DNA or RNA.

The term "tomato plant" includes whole tomato plants, tomato plant organs (e.g., leaves, stems, roots, etc.), seeds and tomato plant cells and progeny of same.

A tomato plant is "derived from" seed or another plant, if it is germinated directly from the seed or is progeny of the plant or seed (e.g., F<sub>1</sub>, F<sub>2</sub>, etc.) as a result of

standard sexual reproduction.

A "primary transformant" is a plant regenerated from one or more plant cells transformed *in vitro* with a recombinant DNA construct.

A "heterologous sequence" is one that originates from a foreign species, or, if from the same species, is substantially modified from its original form. For example, a heterologous promoter operably linked to structural gene is from a species different from that from which the structural gene was derived, or, if from the same species, is different from the promoter normally present with the gene, or is substantially modified from its original form.

Two nucleic acid sequences or polypeptides are said to be "identical" if the sequence of nucleotides or amino acid residues, respectively, in the two sequences is the same when aligned for maximum correspondence as described below. The term "complementary to" is used herein to mean that the sequence is complementary to all or a portion of a reference polynucleotide sequence.

Sequence comparisons between two (or more) polynucleotides or polypeptides are typically performed by comparing sequences of the two sequences over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window", as used herein, refers to a segment of at least about 20 contiguous positions, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned.

Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman *Adv. Appl. Math.* 2: 482 (1981), by the homology alignment algorithm of Needleman and Wunsch *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson and Lipman *Proc. Natl. Acad. Sci. (U.S.A.)* 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, WI), or by inspection. These references are incorporated herein by reference.

"Percentage of sequence identity" is determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or

deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity.

The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 60% sequence identity, preferably at least 80%, more preferably at least 90% and most preferably at least 95%, compared to a reference sequence using the programs described above (preferably BESTFIT) using standard parameters. One of skill will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning and the like. Substantial identity of amino acid sequences for these purposes normally means sequence identity of at least 40%, preferably at least 60%, more preferably at least 90%, and most preferably at least 95%.

Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions. Stringent conditions are sequence dependent and will be different in different circumstances. Generally, stringent conditions are selected to be about 5° C lower than the thermal melting point (T<sub>m</sub>) for the specific sequence at a defined ionic strength and pH. The T<sub>m</sub> is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typically, stringent conditions will be those in which the salt concentration is about 0.02 molar at pH 7 and the temperature is at least about 60°C.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram of the T-DNA region of pWTT2144/AccS.

Figure 2 is a restriction map of the T-DNA region of pWTT2144/AccS showing the location of the hybridization probes used in the genomic mapping.

Figure 3 is a schematic diagram showing the *Acc2* genomic region and *Acc2* cDNA and the fragments used as probes to determine transgene structure in the 1345-4 transformant.

Figure 4 is schematic diagram showing the structure of the T-DNA insert of the invention.

#### DESCRIPTION OF THE PREFERRED EMBODIMENTS

5 The present invention provides tomato plants (*Lycopersicon esculentum*) comprising a stably incorporated locus that confers a delayed fruit ripening phenotype on the plant. In particular, the tomato plants of the invention contain a T-DNA insert comprising a sequence (the AccS transgene) derived from a tomato fruit-specific aminocyclopropane carboxylate (ACC) synthase gene, but that does not encode a  
10 functional ACC synthase enzyme.

ACC synthase is the rate limiting enzyme that converts s-adenosylmethionine to 1-aminocyclopropane-1-carboxylic acid, the immediate precursor to ethylene. As explained in detail below, incorporation of the AccS transgene in a tomato plant genome inhibits expression of the ACC synthase gene. Inhibition of ACC  
15 synthase biosynthesis results in reduced levels of ethylene biosynthesis. Thus, fruit of tomato plants comprising the transgene exhibit a delayed ripening phenotype, but ripen normally when external ethylene is applied.

As used herein an "AccS transgene" is a truncated coding region from the tomato *Acc2* gene which is fused to a 35S promoter from cauliflower mosaic virus and a  
20 *nos3'* termination sequence from *Agrobacterium tumefaciens*. In particular, the sequence from the *Acc2* gene is a sequence consisting of nucleotide 149 to nucleotide 1237. Rottman *et al. J. Mol. Biol.* 222:937-961 (1991).

The plants of the invention are derived, directly or indirectly, from transformation of a tomato plant cell with a construct comprising a T-DNA having the  
25 following regions: (1) LB, the left border region of *Agrobacterium* T-DNA; (2) 2 $\chi$ pnos, a tandem duplicate untranslated promoter region of the nopaline synthase gene from *Agrobacterium*; (3) nptII, the neomycin phosphotransferase gene from Tn5; (4) ocs3', the 3' untranslated region of the octopine synthase gene from *Agrobacterium*; (5) LacZ', an untranslated LacZ polylinker sequence; (6) p35S, the 35S promoter from cauliflower mosaic virus (7) Cab22L, the leader sequence corresponding to the 5' untranslated region  
30 of the Cab22R gene from petunia fused to the 35S promoter; (8) AccS, the truncated coding region from base 149 to base 1237 of the tomato *Acc2* gene; (9) nos 3', the untranslated 3' region of the nopaline synthase gene from *Agrobacterium*; and (10)



RB, a right border region of *Agrobacterium* T-DNA.

The "T-DNA insert" of the invention comprises three copies of the T-DNA described above. The three copies are arranged in inverted repeats at the LB and RB. At the LB-LB and RB-RB junction, one border is deleted such that there is only  
5 one complete border at each junction. The LB and RB at either end of the T-DNA structure are also deleted.

An exemplary plasmid comprising the T-DNA described above, pWTT2144/AccS, is described in detail below. This plasmid is used to generate transgenic tomato plants using *Agrobacterium tumefaciens*-mediated transformation  
10 techniques. Stable insertion of the AccS transgene into the tomato genome in the sense orientation can result in down-regulation of expression of the corresponding endogenous *Acc2* gene and a reduction in ethylene biosynthesis in the ripening fruit. The technique is an example of Transwitch™ suppression (U.S. Patent No. 5,283,184). One feature of Transwitch™ gene suppression is the reduced accumulation of the targeted gene mRNA  
15 (reduced steady state RNA). The trait (level of suppression), once selected through the first sexual generation, behaves in a simple Mendelian fashion.

The methods of the present invention can be used to delay fruit ripening of any tomato (large fruited or cherry) cultivar for fresh market or processing tomato production. Exemplary cultivars that can be used include essentially all commercial  
20 cultivars. For listings of suitable tomatoes see, Rick, in *Evolution of Crop Plants* N.W. Simmonds, ed. pp268-273 (Longman, London, 1976) and Taylor in *The Tomato Crop* pp1-35 (Chapman and Hall, London, 1986).

A tomato plant of the present invention can be obtained by crossing a plant comprising the T-DNA insert of the invention with any tomato cultivar lacking the insert.  
25 Any standard method used for crossing tomato plants can be used to introduce the transgene into the genome of the desired plant. Generally, the methods involve emasculation of one parent, followed by application of pollen from the other parent to the stigma of the first parent. The crosses can be performed using either parent as the pollen parent. Embryo rescue can also be performed if the flowers abort after  
30 pollination.

The plant containing the T-DNA insert can be a plant derived from primary transformants or can be a plant in which the factor was introduced through a sexual cross. Preferred plants of the invention are those derived from seed deposited

with the American Type Culture Collection (ATCC) Accession No. \_\_\_\_\_.

A number of methods can be used to determine if a tomato plant exhibiting a delayed ripening phenotype comprises the T-DNA insert of the invention in its genome. The terms used herein to describe ripeness of tomato fruit are according to standard ripeness classes as described, for instance, in *The Tomato Crop* Atherton and Rudich eds. (Chapman Hall, 1986). The ripeness classes for a given fruit are set forth in Table 1.

Table 1

	Score	Class	Description
10	1	Green	Entirely light to dark-green, but mature.
	2	Breaker	First appearance of external pink, red or tannish-yellow color, not more than 10%.
	3	Turning	Over 10%, but not more than 30% red, pink or tannish-yellow.
	4	Pink	Over 30%, but not more than 60% pinkish or red.
	5	Light red	Over 60%, but not more than 90% red.
15	6	Red	Over 90% red, desirable table ripeness.

Fruit of the plants of the invention reach breaker stage between about 40 and about 70 days after anthesis, usually between about 45 and about 60 days after anthesis. The fruit, however, differ significantly from wild type fruit in terms of ripening inhibition. On the vine, the fruit of plants of the invention typically remain at the breaker stage 5 to 7 days longer than wild type. The transition from breaker stage to light red stage in the ripening impaired fruit is further delayed with respect to the transition for wild type fruit. Under field conditions the fruit of the invention typically require approximately 21 days to proceed from the breaker stage to light red stage, whereas wild type fruit typically require 4 to 5 days to turn from the breaker stage to the red stage. The ripening impaired fruit under field conditions can remain indefinitely at the light red stage without ever reaching the red stage.

There are analogous differences in ripening for off the vine fruit picked at the breaker stage and stored at 15°C. Ripening impaired fruit of the invention typically require at least about 2 weeks (typically about 3 weeks) to reach the pink stage, and while some may reach the light red stage, the fruit can remain indefinitely without

reaching the red stage. Fruit will, however, reach the red stage upon the application of ethylene. This is to be contrasted with wild type fruit which typically require 5 to 7 days to reach red ripe stage from the breaker stage under comparable conditions.

5 Fruit color development, as measured by the a/b ratio measures about 5 to about 10 fold less in the fruit of the invention as compared to control fruit at the breaker or equivalent stage. Standard methods for determining tomato fruit color are described, for instance, in Gull *et al. J. Amer. Soc. Hort. Sci.* 114:950-954 (1989) and Kader *et al. Hort. Sci.* 13:577-578 (1978). At the time the control fruit are red ripe, the levels of color in the fruit of the invention are 4 to 5 times lower.

10 In addition, the fruit do not synthesize normal levels of ethylene during ripening. Typically, the level which is detected at the breaker or equivalent stage is less than about 0.5 nl/g/hr, usually about 0.1 nl/g/hr as measured using a standard assay as described in Grierson and Tucker, *Planta* 157:174-179 (1983) and Sawamura *et al. Plant Cell Physiol.* 19:1061-1069 (1978). At the pink stage which follows the breaker stage  
15 the fruit continue to have ethylene levels which are reduced by about 50 to about 100 fold compared to control fruit.

Since the T-DNA inserts comprise NPTII genes, kanamycin resistance can be determined using selective media or by spraying 10-14 day old tomato seedlings with a solution of kanamycin (1g/L) on three consecutive days. The levels of *Acc2* mRNA  
20 can be measured using standard techniques such as Northern blots, RNase protection assays and the like. To further characterize the plants nucleic hybridization techniques can be used to determine structure of the T-DNA insert in the plant. The example section, below, provides a detailed description of the molecular characterization of the T-DNA insert of the invention.

25 The precise locus in which the T-DNA insert of the invention is integrated can be determined using standard genetic and molecular mapping techniques well known to those of skill in the art. Obviously, for plants derived either directly or indirectly from a particular plant or seed containing the T-DNA insert (*e.g.*, those deposited with the ATCC under Accession No. \_\_\_\_\_), the locus will be the same as the parent plant.

30 The following example is provided to illustrate, but not limit the claimed invention.

#### Example 1

##### A. Agrobacterium-mediated transformation system

Introduction of DNA into plant tissue by *Agrobacterium*-mediated transformation as described in U.S. Patent No. 5,283,184. The vector system used to transfer the AccS transgene into tomato is based on the Ti plasmid from *Agrobacterium tumefaciens*.

5           The T-DNA plasmid, pWTT2144/AccS, used in these transformations is composed of: (1) the replication of origin from pACYC184 that ensures replication in *Escherichia coli*; (2) the pVS1 replicon (derived from *Pseudomonas aeruginosa* DNA) that ensures replication in *A. tumefaciens*; (3) the tetracycline resistance marker from plasmid RP1 that allows for selection of the binary plasmid in *A. tumefaciens* and *E.*  
10 *coli*, and (4) the left and right border regions of T-DNA from an octopine strain of *A. tumefaciens* which surround the DNA insertion in the plant genome.

          Within the T-DNA are the *nptII* gene from transposon Tn5 that encodes enzyme neomycin phosphotransferase II and serves as a selectable marker for transformed plant cells, fused to a nopaline synthase (*nos*) promoter sequence and  
15 octopine synthase (*ocs3'*) termination sequence from *A. tumefaciens*, and the LacZ' polylinker region with multiple restriction sites for cloning of genes to be transferred. The T-DNA has an insertion of a truncated *Acc2* gene coding region fused to the 35S promoter from cauliflower mosaic virus and the *nos3'* termination sequence in the LacZ' polylinker region of pWTT2144.

20           Plasmid pWTT2144 was transferred from *E. coli* to *A. tumefaciens* LBA4404, which carries the pAL4404 *vir* plasmid, by a triparental mating procedure as described by Figurski *et al.*, *Proc. Natl. Acad. Sci. USA* 76:1648-1652 (1979).

          The plasmid pWTT2144/AccS was used to transform the parental line 91103-114 to generate line 1345-4, described in detail below. The T-DNA region of this  
25 plasmid consists of the following sequences (*see*, Figure 1):

CaMV35S. The 35S promoter region is derived from cauliflower mosaic virus and controls expression of the AccS gene. The 35S promoter directs high level constitutive expression and is widely used as a promoter for high expression of  
transgenes.

30           Cab22L leader. The Cab22L leader sequence (Cab22L) is a 69 bp fragment of *Petunia hybrida* genomic DNA which was derived from the Cab22L gene and corresponds to the 5' untranslated region for that gene.

AccS. The AccS gene is a truncated coding region derived from an ACC

synthase gene (*Acc2*) isolated from tomato (*L. esculentum*). The *AccS* gene corresponds to a 1088 bp region of the *Acc2* gene from base 149 to base 1237. The *AccS* gene does not encode a functional ACC synthase enzyme.

Termination sequences. The nopaline synthase (*nos3'*) and octopine synthase (*ocs3'*) gene termination sequences from *A. tumefaciens* function in the expression of the *AccS* and *nptII* genes, respectively.

2Xpnos Promoter. The *nos* promoter is present in line 1345-4 as a duplicate tandem repeat of the untranslated 5' region of the nopaline synthase gene from *A. tumefaciens*. It functions in line 1345-4 in the expression of the *nptII* selectable marker gene. This sequence, as used in line 1345-4, no longer functions as a regulated article since it is not associated with the nopaline synthase coding region which functions in *A. tumefaciens*.

NptII. The *nptII* gene is a coding region originally isolated from transposon Tn5. It encodes a protein, neomycin phosphotransferase II, which catalyzes the phosphorylation of certain aminoglycoside antibiotics, rendering transformed cells resistant to kanamycin. It functions in line 1345-4 as a selectable marker.

LacZ' polylinker sequence. The untranslated LacZ' polylinker sequence functions in line 1345-4 as a site for cloning the *AccS* transgene into the binary vector pWTT2144.

Borders. The left and right border regions of T-DNA from *A. tumefaciens* function in the transfer of gene sequences into the tomato genome. The border regions are the only necessary *cis*-acting elements in T-DNA for T-DNA insertion. The use of a binary vector system allows for other necessary transfer elements to act in *trans* so that only the border regions are required to be integrated into the plant host genome. The T-DNA borders are only partially transferred to the tomato genome; during the transformation process, the left border is cut between nucleotides 293 and 294 (left border nick) while the right border is cut between nucleotides 7603 and 7604 (right border nick). This cleavage reduces the length of the right border fragment of pWTT2144/*AccS* from 1900 bp to 303 bp in the T-DNA and the left border fragment of pWTT2144/*AccS* from 880 bp to 589 bp in the T-DNA (Figure 1).

#### **B. Description of Non-transformed Tomato Cultivar 91103-114**

DNAP tomato line 91103-114 is a somaclone derived from the breeding line FL7181 developed by Dr. Jay Scott at the University of Florida. Line FL7181 is characterized as a determinate large-fruited variety with an average fruit weight of 8 oz.

Fruit are globose to slightly elliptical in shape and are substantially firmer than fruit of comparable varieties (i.e., Floradade). Fruit shoulders are smooth and exhibit a darker green shade than the rest of the fruit surface before ripening. Fruit ripen to a deep crimson red interior color due to the presence of the *og'* allele. The fruit stem (pedicel) lacks a joint. This line is known to be resistant to Verticillium wilt race 1 and Fusarium wilt races 1 and 2.

The DNAP line 91103-114 exhibits all of the traits described above, but differs from FL7181 principally by reduced blossom end scar size, strong main stem and increased foliage cover for fruit. Line 91103-114 also differs from FL7181 in its adaptation to diverse growth environments; while FL7181 is specifically adapted to Florida growing regions, 91103-114 has proven to grow well in other regions (e.g., California).

**C. Description, History and Mendelian Inheritance of Delayed-Ripening Tomato Line 1345-4**

The line 1345-4 is a homozygous T<sub>2</sub> selection from an original T<sub>0</sub> transformant 1345, obtained after *Agrobacterium tumefaciens* transformation of the DNAP line 91103-114 with binary vector pWTT2144/AccS.

The primary transformant 1345 was selected in a greenhouse screen of several hundred primary transformants to have fruit which did not ripen when left on the vine. Subsequently, 10 of the 1345 T<sub>1</sub> seed (derived from self fertilization) which were prescreened for kanamycin resistance were screened in the greenhouse and observations on the plant and fruit phenotype were made. Plant 1345-4 was selected as having fruit which did not ripen on the vine. The 1345-4 plant was shown to be homozygous for the T-DNA locus. Seed from the self-fertilization of 1345-4 have been subsequently analyzed in multiple field trials over multiple growing seasons.

No instability in the delayed-ripening phenotype has been observed in any field trials. Two independent ways have been used to monitor the stability of line 1345-4. First, it is possible to establish that the T-DNA insertion is stable and intact by measuring kanamycin resistance in large populations of seedlings in the greenhouse. It was established that the 1345 plant was carrying a single T-DNA insertion by evaluating the segregation of kanamycin resistance in the primary transformant. Subsequently, multiple kanamycin-resistant T<sub>2</sub> plants were selected and segregation of the kanamycin-resistant phenotype in progeny plants arising from self-fertilization of the

selected T2 plants was evaluated. The T3 plants from 1345-4 were all kanamycin resistant, hence it was deduced that the 1345-4 plant was homozygous.

The second approach to evaluating the stability of line 1345-4 is through observation of the delayed-ripening phenotype in the field. A number of separate field trials for the evaluation of the homozygous 1345-4 line and progeny derived from it have been carried out. These trials have involved the evaluation of fruit on at least 2000 separate plants of 1345-4. During these evaluations, which involved observations of the individual plants at multiple times during the ripening process and the harvest of fruit from all of the plants, no exceptional plants in which all the fruit ripen at the normal rate have been observed.

#### **D. DNA Analysis of Delayed-Ripening Tomato Line 1345-4**

To determine the nature and number of insertions which have occurred in line 1345, Southern hybridizations were used to characterize the structure of the T-DNA inserts in the genomic DNA, in conjunction with the *np111* segregation data described above which indicates the T-DNA locus number. The T-DNA is defined as the region between the left and right borders of the binary vector pWTT2144/AccS that is transferred into the plant (see Figure 1). This region includes the *np111* selectable marker and the truncated ACC synthase gene (AccS), together with the left and right border sequences. Figure 2 shows the restriction enzymes used to cleave the DNA and the location of the four probes used to determine the structure of T-DNA insert, the left border (LB), *np111*, *Acc2*, and right border (RB)-probes.

##### **1. Copy number**

The number of additional AccS genes in 1345 was determined by digesting genomic DNA from transgenic plants with HindIII and EcoRI, then after electrophoresis and transfer to nylon membranes, hybridizing to a <sup>32</sup>P-labeled *Acc2* probe. This probe hybridizes to a 0.9 kb EcoRI-HindIII fragment from the transgene (AccS) and a 1.2 kb HindIII fragment from the endogenous *Acc2* gene which includes an additional 300 bp of intron sequence. By comparing the intensity of the transgene and endogenous bands, a determination of the number of copies of the transgene can be made in either the hemizygous primary transformant or homozygous S1 progeny. The hybridization patterns of genomic DNA from an untransformed tomato plant, Baxter's Early Bush Cherry (BEB) and 1345-4 digested with HindIII and EcoRI and hybridized to the *Acc2* probe were analyzed. In 1345-4, the homozygous S1 progeny of 1345, the

endogenous gene to transgene ratio is greater than 2 suggesting that there are at least 2 copies of AccS in the transgenic DNA. Since the *np11* gene segregates as a single locus, it is most probable that the 2-3 copies of the T-DNA are present at a single locus.

## 2. T-DNA Structure

5 It is known that a single intact copy or multiple T-DNA copies can be inserted at a single locus as direct or inverted repeats around either the left or right border, and it is known that deletions of the T-DNA or insertions of genomic DNA may be present between the T-DNA copies (Jorgenson *et al.*, *Mol. Gen. Genet.* 207:471-477 (1987)). To determine the organization of the T-DNAs in the 1345 genome, we  
10 hybridized several different probes to the 1345-4 genome digested with several restriction enzymes. Figure 2 shows the relative map position of the probes in the T-DNA.

*Left and Right Borders:* EcoRI digestions were done to determine the number of intact left and right border fragments. EcoRI sites in the T-DNA are located approximately 500 bp in from the LB and 2.1 kb in from the RB (within the AccS  
15 transgene). The fragments hybridizing to the specific border probe will be at least this size. If there is an inverted repeat at the LB and the borders are intact and flush, we would expect to see a 1 kb EcoRI fragment hybridizing only to the LB. For the RB, an intact inverted repeat would give a 4.2 kb fragment that hybridizes to the RB fragment as well as to the AccS probe. A direct LB-RB repeat would result in a fragment of 2.6 kb  
20 that hybridizes to both border probes and AccS.

In 1345-4 DNA cut with EcoRI, a 2.9 kb fragment hybridizes to the LB probe only and a 3.5 kb fragment hybridizes to the RB probe only, indicating that there is one intact left and right border. Likewise, the NcoI digestions show single hybridizing bands with both probes and the fragments are of appropriate sizes (LB fragment, greater  
25 than 1.6 kb and RB fragment greater than 2.3 kb). The HindIII and XbaI digests also show single bands of appropriate sizes which suggests that there is a single complete T-DNA insertion. However, we know from the copy number blots (see above) that there are at least two copies of the AccS transgene. Together these results suggest that a deleted form of T-DNA, containing an intact AccS gene but missing one or both of the  
30 borders, is also present. There are no direct repeat structures since the LB and RB probes do not hybridize to the same fragment. There could, however, be inverted or indirect repeats around either border. Hybridizations with the *np11* and AccS probes were done to further characterize the T-DNA insertions.



*nptII*: If the T-DNA is intact, the *nptII* probe will hybridize to a single 2.4 kb EcoRI fragment containing the entire 2Xpnos-*nptII*-ocs3' fusion (see Figure 2). In 1345-4 genomic DNA cut with EcoRI, the expected 2.4 kb fragment is present in addition to two other hybridizing fragments of 5.2 kb and 2.9 kb. The 2.9 kb fragment also hybridizes to the left border probe which indicates that there is probably one complete internal copy and 2 incomplete copies of T-DNA with deletions occurring at the LB. Since the 2.9 kb EcoRI fragment hybridizes to both *nptII* and LB probes, this junction is likely an inverted repeat with a deletion of one of the borders that includes the EcoRI site. Hybridization of an EcoRI fragment to both the LB probe and the *nptII* probe can only occur if a deletion eliminates one of the EcoRI sites.

Since NcoI cleaves within the *nptII* coding sequence, digestion of the T-DNA with this enzyme will give two *nptII* fragments, one of 2.1 kb which spans the *nptII* 3' coding region up to the NcoI site located at the ATG of the AccS transgene, and one of 1.2 kb in length which includes the 5' *nptII* coding region and LB to the next NcoI site in either genomic DNA or adjacent T-DNA insertion. If there is a perfect inverted repeat at the LB, we expect a 3.3 kb NcoI fragment that hybridizes to both the *nptII* and LB probes. Based on the results from the EcoRI digestion, we expect the 2.1 kb fragment to be present, as well as two fragments greater than 1.2 kb. One of these will also hybridize to, the LB. As predicted, the 2.1 kb fragment is present as well as a 6.6 kb fragment and a 2.9 kb fragment which also hybridizes to the LB probe. This is consistent with the presence of three T-DNA copies, one which is complete and intact, one which is an inverted repeat with a deletion (approximately 600 bp) extending to the LB, and a second inverted repeat with a deletion (approximately 50 bp) extending to the right border.

*AccS*: Hybridization with the AccS probe will give fragments greater than 2.3 kb and 2.1 kb for NcoI and EcoRI digests respectively. These fragments will also hybridize to the RB probe if the border is intact. The results show two hybridizing fragments for NcoI, 15 kb and 3.8 kb, and two for EcoRI, 4 kb and 3.5 kb. The 3.8 kb NcoI fragment and the 3.5 kb EcoRI fragment also hybridize to the RB. These results confirm that there are at least 2 copies of AccS, and that one copy is present on a T-DNA with a deleted RB. To demonstrate that AccS itself has not been deleted or rearranged, NcoI/XbaI double digests were done to drop out the intact 1.1 kb fragment containing the complete truncated gene.

*Linkage of AccS and nptII:* XbaI and HindIII cleave the T-DNA once approximately 1 kb in from the right border. Digestion with either of these enzymes will generate fragments containing both the *nptII* and *AccS* genes. After XbaI digestion, a 9.2 kb and a 6.2 kb fragment hybridized with both probes. After HindIII digestion, a 9.2 kb and 6.4 kb fragment hybridized to both probes confirming that each copy of *AccS* is linked to a copy of *nptII*. The 9.2 kb fragment also hybridizes to the LB. The size of the fragments are consistent with the presence of inverted T-DNA repeats at the LB and RB.

The structure for 1345-4 T-DNA insertion is shown in Figure 4. It consists of three T-DNAs assembled in inverted repeats at the LB and RB. At the LB-LB and RB-RB junction, one border is deleted such that there is likely only one complete border at each junction. The LB and RB at either end of the T-DNA structure are also deleted. The endpoint rightward is internal to the *nptII* gene and deletes the internal NcoI site, and the endpoint leftward lies between the *Acc2* gene and the right border.

Finally, the nucleotide sequence of the T-DNA insert of 1345-4 has been compiled from the deduced restriction map and is present in SEQ. ID. No. 1.

The above examples are provided to illustrate the invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference.

SEQ. ID. No. 1

## 1345-4 T-DNA Locus

LacZ <- | -> nos3'

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TCTATCGCGTATTAAATGTATAATTGCGGGACTCTAATCATAAAAACCCA  
TCTCATAAATAACGTCATGCATTACATGTTAATTATTACATGCTTAACGT  
AATTC AACAGAAATTATATGATAATCATCGCAAGACCGGCAACAGGATTC  
AATCTTAAGAACTTTATTGCCAAATGTTTGAACGATCTGCCCCCTTGACT  
nos3' <- |  
-> ACCS  
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-> 358Cab22L  
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358Cab22L<-|->LacZ

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LacZ<-|->ocB3'

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GCTCAGGTTTTTTTACAACGTGCACAACAGAATTGAAAGCAAATATCATGC

ocs3' &lt;- | -&gt; NPTII

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1-7 2:00

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pnos ← LB / LB deleted

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LB/LB deleted<-|->pnos

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|->pnos

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pnos<-|->NPTII

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NPTII<-|->oc83'

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ocs3' <- | -> lacZ

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lacZ <- | -> 35Scab22L

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35Scab22L<-|  
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nos3'|->lacZ  
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nos3'<-|  
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->RB/deleted RB

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RB/deleted RB<-|->lacZ

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lacZ<-|

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->nos3'

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nos3'<-|->ACCS

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GACTCTAAATCCTGGTAACCCCATGTCTTTTGAAAGACTGTAGACGATGT  
GAACTAAATCTTTGTTGCAGTAAGTCATTTCTGTTCATCGAGGATTTCA  
GCTATACTGACGAATTGAGGCGTGTCAAAGACAGTGGCTGCGTAGATTTTC  
GTCACAAACAAGGTGGATGTTGTGTTGGTTGGTGAAACTCAAGACACTTT  
TCAGTGTGTCTTTGTCCAAAGTGGTGCCCAATGGATTGTGATGGATTGGTC

AAAATCAAACCTTTTACTTTGATGTTTGATTTTTGTGCATTTTCATATGC  
TTCTTTTACTGCTTTTGAAGTAATTTTGAAATTATTGGAGCTCTCACAGT  
GAATTGGAATAAGTTGTACTCCAGTTCTCCATCTTAAATCTCTGTTAAAT  
GCTGGGTAGTATGGTGAAGGTACTAAAAATGCATCGCCAGGATCAGCCAA  
ACAAAATATAATTGTCTCATTAGCTCCAGTGGCACCACCAGCCATAACAA  
CTCTTTCTGGATCAAATCTAACTCTTCCTCCTCTTGTTTTCTCCATAAAT  
TTCGCAATCGCTTTTCTGAATTCAGGCAAGCCATGATAATCTTGAAAGTT  
GGCAATGGCCTTGAATGATTTGATTCCCTCAGAACAAATTGAACCTTTTG  
GGTTTCTCTTAATCCAATCTTCTATCAAGTCTAAACAAAGCTGATTTTCT

**ACCS<-|->35Scab22L**

GCTAAACCCATGGTTTAAATAAGAAGAGAAAAGAGTTCTTTTGTTATGGCT  
GAAGTAATAGAGAAATGAGCTCGAGCGTGTCTCTCCAAATGAAATGAAC  
TTCCTTATATAGAGGAAGGGTCTTGCGAAGGATAGTGGGATTGTGCGTCA  
TCCCTTACGTCAGTGGAGATGTCACATCAATCCACTTGCTTTGAAGACGT  
GGTTGGAACGTCTTCTTTTCCACGATGCTCCTCGTGGGTGGGGGTCCAT  
CTTTGGGACCACTGTCCGCAGAGGCATCTTGAATGATAGCCTTTCTTTA  
TCGCAATGATGGCATTGTAGGAGCCACCTTCCTTTTCTACTGTCCTTTC  
GATGAAGTGACAGATAGCTGGGCAATGGAATCCGAGGAGGTTTCCCGAAA  
TTATCCTTTGTTGAAAAGTCTCAATAGCCCTTTGGTCTTCTGAGACTGTA  
TCTTTGACATTTTGGAGTAGACCAGAGTGTCGTGCTCCACCATGTTGAC  
GAAGATTTTCTTCTTGTCATTGAGTCGTAAAAGACTCTGTATGAACTGTT  
CGCCAGTCTTCACGGCGAGTTCTGTTAGATCCTCGATTTGAATCTTAGAC  
TCCATGCATGGCCTTAGATTCACTAGGAACTACCTTTTATAGAGACTCCAA  
TCTCTATTACTTGCCTTGGTTTATGAAGCAAGCCTTGAATCGTCCATACT  
GGAATAGTACTTCTGATCTTGAGAAATATGTCTTCTCTGTGTTCTTGAT  
GCAATTAGTCCTGAATCTTTTGACTGCATCTTTAACCTTCTTGGAAGGT  
ATTTGATCTCCTGGAGATTGTTACTCGGGTAGATCGTCTTGATGAGACCT  
GCTGCGTAGGCCTCTCTAACCATCTGTGGGTGAGCATTCTTTCTGAAATT  
GAAGAGGCTAACCTTCTCATTATCAGTGGTGAACATAGTGTCGTACCTT  
CACCTTCGAACCTTCCTTCCTAGATCGTAAAGATAGAGGAAATCGTCCATT

**35Scab22L<-|->lacZ**

GTAATCTCCGGGGCAAAGGAGATCCCGGTACCGAGCTCGAATTCGTAAT  
CATGGTCATAGCTGTTTCTGTGTGAAATTGTTATCCGCTCACAATTCCA  
CACAACATACGAGCCGGAAGCATAAAGTGTAAGCCTGGGGTGCCTAATG  
AGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGT  
CGGGAAACCTGTGCGTCCAGCTGCATTAATGAATCGGCCAACGCGCGGGG

lacZ<-|->ocs3'

AGAGGCGGTTTGCCTATTGGGCAGCGGCCGCTGTTACCCGGCCGCCGTGC  
TGGACCGGGTTGAATGGTGGCCGTAACCTTTCGGTAGAGCGGACGGCCAAT  
ACTCAACTTCAAGGAATCTCACCCATGCGCGCCGGCGGGGAACCGGAGTT  
CCCTTCAGTGAACGTTATTAGTTTCGCCGCTCGGTGTGTCGTAGATACTAG  
CCCCTGGGGCCTTTTGAAATTTGAATAAGATTTATGTAATCAGTCTTTTA  
GGTTTGACCGGTTCTGCCGCTTTTTTTTAAAATTGGATTTGTAATAATAAA  
ACGCAATTGTTTGTATTGTGGCGCTCTATCATAGATGTGCTATAAACC  
TATTCAGCACAAATATATTGTTTTTCATTTTAATATTGTACATATAAGTAGT  
AGGGTACAATCAGTAAATTGAACGGAGAATATTATTCTATAAAAATACGAT  
AGTAACGGGTGATATATTCATTAGAATGAACCGAAACCGGCGGTAAGGAT  
CTGAGCTACACATGCTCAGGTTTTTTTACAACGTGCACAACAGAATTGAAA

ocs3'<-|->NPTII

GCAAATATCATGCGATCATAGGCGTCTCGCATATCTCATTAAAGCAGGGG  
GTGGGCGAAGAACTCCAGCATGAGATCCCCGCGCTGGAGGATCATCCAGC  
CGGCGTCCCGGAAAACGATTCCGAAGCCCAACCTTTCATAGAAGGCGGCG  
GTGGAATCGAAATCTCGTGATGGCAGGTTGGGCGTCGCTTGGTGCGTCAT  
TTCGAACCCCGAGTCCCGCTCAGAAGAACTCGTCAAGAAGGCGATAGAA  
GGCGATGCGCTGCGAATCGGGAGCGGCGATACCGTAAAGCACGAGGAAGC  
GGTCAGCCCATTCCGCCGCAAGCTCTTCAGCAATATCACGGGTAGCCAAC  
GCTATGTCTTGATAGCGGTCCGECACACCCAGCCGGCCACAGTCGATGAA  
TCCAGAAAAGCGGCCATTTTCCACCATGATATTCCGCAAGCAGGCATCGC  
CATGGGTACGACGAGATCCTCGCCGTCGGGCATGCGCGCCTTGAGCCTG  
GCCAACAGTTCGGCTGGCGCGAGCCCCTGATGCTCTTCGTCCAGATCATC  
CTGATCGACAAGACCGGCTTCCATCCGAGTACGTGCTCGCTCGATGCGAT  
GTTTCGCTTGGTGGTGAATGGGCAGGTAGCCGGATCAAGCGTATGCAGC  
CGCCGCATTGCATCAGCCATGATGGATACTTTCTCGGCAGGAGCAAGGTG  
AGATGACAGGAGATCCTGCCCCGGCACTTCGCCCAATAGCAGCCAGTCCC  
TTCCCGCTTCAGTGACAACGTCGAGCACAGCTGCGCAAGGAACGCCCGTC  
GTGGCCAGCCACGATAGCCGCGCTGCCTCGTCTGTCAGTTCATTTCAGGGC  
ACCGGACAGGTGCGTCTTGACAAAAGAACCGGGCGCCCCCTGCGCTGACA  
GCCGGAACACGGCGGCATCAGAGCAGCCGATTGTCTGTTGTGCCAGTCA  
TAGCCGAATAGCCTCTCCACCCAAGCGGCCGGAGAACCTGCGTGCAATCC

NPTII<-

ATCTTGTTCAATCATGCGAAACGATCCTCATCCTGTCTCTTGATCAGATC

WHAT IS CLAIMED IS:

1. A tomato plant comprising a genetic locus having a sequence substantially identical to SEQ. ID. No. 1.

5                   2. The plant of claim 1, wherein the plant bears fruit that reach pink stage about 2 to about 3 weeks after breaker stage, when the fruit are picked at breaker stage and stored at 15°C.

10                   3. The plant of claim 1, wherein the fruit do not reach red stage in the absence of application of ethylene.

                  4. The plant of claim 1, which is germinated from seed deposited with the American Type Culture Collection under Accession No. \_\_\_\_\_.

15                   5. A tomato fruit from the plant of claim 1.

                  6. A tomato fruit that reaches pink stage about 2 to about 3 weeks after breaker stage, when the fruit is picked at breaker stage and stored at 15°C.

20                   7. The tomato fruit of claim 6, wherein the fruit does not reach red stage in the absence of application of ethylene.

                  8. The tomato fruit of claim 6, wherein the fruit has an ethylene level less than about 1.0 nl/g/hr.

25                   9. A method of making a tomato plant with decreased ethylene production, the method comprising:

                  crossing a parent tomato plant with a tomato plant comprising a genetic locus having a sequence substantially identical to SEQ. ID. No. 1, thereby producing  
30 progeny.

                  10. The method of claim 9, wherein the tomato plant comprising the genetic locus is germinated from seed deposited with American Type Culture Collection

under Accession No. \_\_\_\_\_.

11. The method of claim 9, further comprising the step of selecting  
comprises selecting progeny bearing fruit that reach pink stage about 2 to about 3 weeks  
after breaker stage, when the fruit are picked at breaker stage and stored at 15°C.

12. A plant made according to the method of claim 9.

13. A tomato plant comprising a genetic locus comprising:  
an inserted T-DNA, consisting essentially of, from the 5' to the 3'  
direction, a left border sequence, a 2Xpnos promoter sequence, an NPTII gene, an ocs3'  
untranslated region, a p35S promoter sequence, a sequence consisting of nucleotide 149  
to nucleotide 1237 of a tomato Acc2 gene, a nos3' untranslated region, and a right  
border sequence;

a right border inverted repeat of the T-DNA linked to the right border  
sequence, the right border inverted repeat lacking a left and a right border sequence; and

a left border inverted repeat linked to the left border sequence, the left  
border inverted repeat lacking a left and a right border sequence.

14. A tomato fruit from the plant of claim 13.

15. A tomato plant which is germinated from seed deposited with the  
American Type Culture Collection under Accession No. \_\_\_\_\_.

16. A tomato fruit from the plant of claim 15.

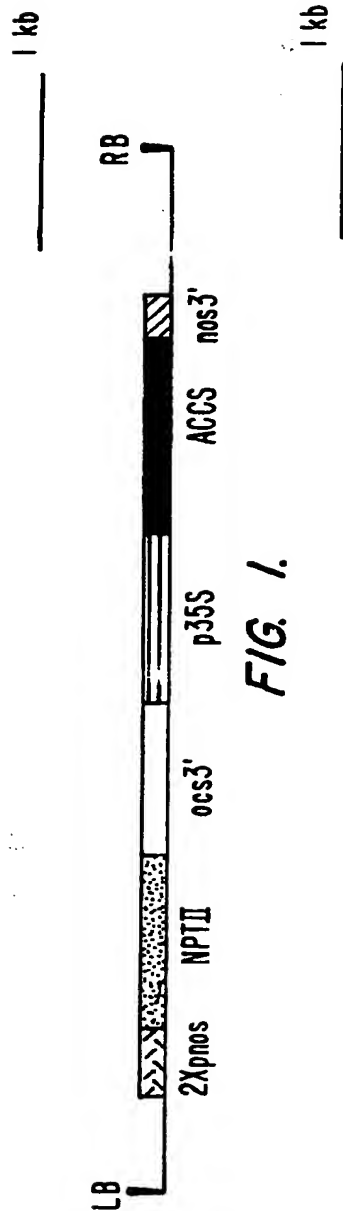


FIG. 1.

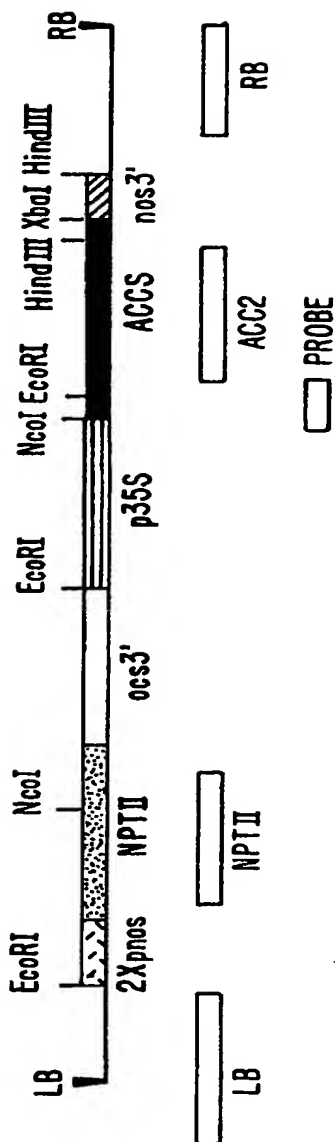


FIG. 2.

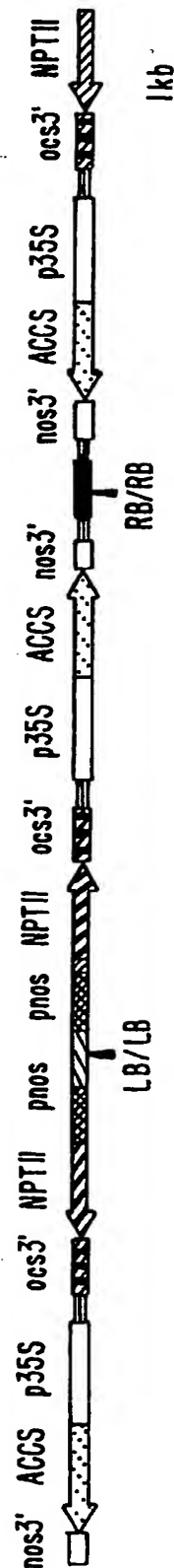
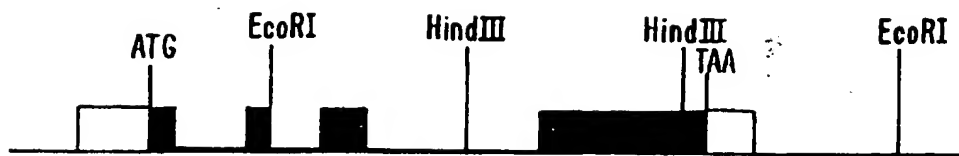


FIG. 4.



2/2

ACC2







 1.2 kb HindIII  
FRAGMENT

C2 cDNA



0.9 kb HindIII - EcoRI  
FRAGMENT

-  UNTRANSLATED REGION
-  EXON (CODING SEQUENCE)
-  HYBRIDIZING FRAGMENT
-  INTRON

(NOT TO SCALE)

**FIG. 3.**

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/11096

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A01H 1/04, 4/00, 5/00, 5/08

US CL : 800/200, 205; 435/172.3; 47/58

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/200, 205, DIG 44; 435/172.3; 47/58

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE, APS  
search terms: ACC2

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ROTTMANN et al. 1-Aminocyclopropane-1-Carboxylate Synthase in Tomato is Encoded by a Multigene Family Whose Transcription is Induced During Fruit and Floral Senescence. J. Mol. Biol. 1991, Volume 222, pages 937-961.	6-8 and 13-14
Y	VAN DER STRAETEN et al. Cloning, genetic mapping, and expression analysis of an Arabidopsis thaliana gene that encodes 1-aminocyclopropane-1-carboxylatesynthase. Proc. Natl. Acad. Sci. USA. October 1992, Volume 89, pages 9969-9973.	6-8 and 13-14

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be part of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

17 SEPTEMBER 1996

Date of mailing of the international search report

10 OCT 1996

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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/11096

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LIANG et al. The 1-aminocyclopropane-1-carboxylate synthase gene family of Arabidopsis thaliana.. Proc. Natl. Acad. Sci. USA, November 1992, Volume 89, pages 11046-11050.	6-8 and 13-14
Y	TIGCHELAAR et al. 'Use of tomato fruit ripening mutants to enhance fruit storage life.' In: Tomato and pepper production in the tropics: international symposium on integrated maagement practices. Edited by Mclean et al. Taiwan, 1989, pages 123-136.	6-8
Y	US, A, 4,843,186 (NAHUM) 27 June 1989, see the entire document.	6-8

# INTERNATIONAL SEARCH REPORT

In ational application No.  
PCT/US96/11096

## B x I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 1-5, 9-12, and 15-16  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  
  
Claim nos. 1-5, 9-12 and 15-16 have not been searched because Applicants have not provided a CRF in compliance with the sequence rules (claims 1-5, 9-12) and have not provided an ATCC accession No. (claims 4, 10, and 15-16).
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.